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Abstract

Limbal epithelial stem cells play a key role in the maintenance and regulation of the corneal surface. Damage or destruction of these cells results in vascularisation and corneal opacity. Subsequent limbal stem cell transplantation requires an ex vivo expansion step and preserving cells in an undifferentiated state remains vital. In this report we seek to control the phenotype of limbal epithelial stem cells by the novel application of compressed collagen substrates. We have characterised the mechanical and surface properties of conventional collagen gels using shear rheology and scanning electron microscopy. In doing so, we provide evidence to show that compressive load can improve the stiffness of collagen substrates. In addition Western blotting and immunohistochemistry display increased cytokeratin 3 (CK3) protein expression relating to limbal epithelial cell differentiation on stiff collagen substrates. Such gels with an elastic modulus of 2900 Pa supported a significantly higher number of cells than less stiff collagen gels (3 Pa). These findings have substantial influence in the development of ocular surface constructs or experimental models particularly in the fields of stem cell research, tissue engineering and regenerative medicine.

Keywords: Limbal Stem Cells, Collagen Gel, Differentiation

Abbreviations: LEC, limbal epithelial cells; SEM, scanning electron microscopy; CK3, cytokeratin 3; CK14, cytokeratin 14; ZO1, zonula occludens-1.

Introduction

It is well known that the behaviour of cells can be regulated by biochemical and topographical cues (Cheng et al., 2011; Jayawarna et al., 2009; L. H. Lee et al., 2009; Liu et al., 2007; Waese & Stanford, 2011; Zhou et al., 2009). Most recently it has been shown that multipotent human mesenchymal stem cells are sensitive to the elasticity of substrates which when altered can give rise to specific cell lineages, (Engler et al., 2006; Guo et al., 2006). We seek to apply this concept to control the fate of unipotent limbal epithelial stem cells by replacing synthetic substrates with a naturally occurring material. In our model the substrates' (conventional collagen gels) mechanical properties are controlled by compressive load. Using this approach we have evaluated a model by which the phenotype of limbal epithelial cells can be controlled. This approach opens up the possibility of engineering better defined tissue constructs for ocular surface regenerative medicine.

Adult stem cells of the cornea reside within an anatomical region at the edge of the cornea termed the limbus; as such limbal epithelial cells (LEC) are important for the maintenance and regulation of the corneal surface (Cotsarelis et al., 1989; Lehrer et al., 1998). Destruction or damage to the corneal limbus and the stem cells concentrated therein may lead to blindness via vascularisation or stromal opacity of the cornea (Grueterich et al., 2003). The ex vivo expansion of limbal stem cells is an important step in the current treatment of corneal blindness caused by total limbal stem cell deficiency (Grueterich et al., 2003; Tseng et al., 1998) and once expanded in culture the cells are transplanted on to the corneal surface either with or without the substrate. Limbal stem cells have been successfully expanded upon a range of different substrates including amniotic membrane, temperature responsive polymers,

contact lenses, collagen and fibrin gels (Di Girolamo et al., 2007; Koizumi et al., 2000; Mi et al., 2010b; Nishida et al., 2004; Rama et al., 2001).

Collagen is a major component of the extracellular matrix and subsequently one of the most abundant structural proteins within mammalian connective tissue. Within the cornea, the stroma comprises predominantly of type I and V collagens (Newsome et al., 1982) and lies below the corneal epithelium. Here collagen fibrils are highly aligned and packed in tight sheets. This ordered arrangement of collagen accounts for the transparency of the cornea (Maurice, 1957). As a substrate collagen is highly compatible with low levels of immunogenicity (Bell et al., 1979) making it an excellent structure for tissue engineering applications. Therefore collagen based substrates are desirable for tissue engineering ocular surface models.

Having previously shown that collagen gels can mimic the structure of biological tissues and, when combined with corneal cells, form a functional ocular surface construct (Mi et al., 2010a; Mi et al., 2010b; Mi et al., 2011); we have now sought to investigate the influence of collagen gel stiffness on LEC phenotype. We show that stiff collagen gels with dense and compacted collagen fibres facilitate the differentiation of LEC. These findings will facilitate the development of well-defined tissue constructs for future stem-cell-based regenerative medicine.

Results and Discussion

Subsequent to our previous findings (Mi et al., 2010b) we were able to further improve both the compaction of collagen fibres by compressing thicker collagen gels, and the surface structure. Scanning electron microscopy was employed to characterise the nano-structure of our stiff compressed and less stiff uncompressed collagen gels **(Fig. 1)** and we found that the compaction of collagen fibrils on the surface of both materials were dissimilar in terms of collagen fibre arrangement. It was evident that both compressed and uncompressed collagen gels possessed different surface features in terms of nano-fibrillar morphology and arrangement. This is shown by differences in contrast on the surface of the uncompressed collagen gel **(Fig. 1c)** where lighter areas are attributed to an irregular or rough surface. Hence plastic compaction of the collagen fibres in the compressed collagen gel improved surface topography.

In addition to characterising the surface nanostructure of our substrates, oscillatory shear rheology was employed to compare the viscoelastic properties of compressed and uncompressed substrates where gel stiffness, or elastic modulus (G′) was quantified. Measurements show that there was a distinct difference between the storage moduli of compressed and uncompressed collagen gels as indicated by stress sweeps **(Fig. 2a)**. Following cell expansion these values remained significantly different (30 Pa and 1500 Pa for uncompressed and compressed collagen gels respectively). Compressed collagen gels were found to be within the linear viscoelastic regime for oscillatory stresses up to 20 Pa whilst the linear regime for uncompressed collagen gels was substantially shorter, up to 2 Pa after which there was a steady decline in elastic modulus at higher oscillatory stresses. Therefore fixed

stress values of 10 Pa and 1 Pa were selected for frequency sweep studies corresponding to stresses within the linear viscoelastic regime of the compressed and uncompressed collagen gels respectively. **Figure 2b** shows frequency sweep data to further characterise the structure of our scaffolds. Uncompressed collagen substrates behave as weak gels with low values of G′. In comparison the elastic modulus of compressed collagen substrates is significantly higher ($G' > 10^3$ Pa) showing weak frequency dependence, typical behaviour of a strong gel. Uncompressed collagen gels show frequency dependence from around 5 $rads^{-1}$ and so the mechanical properties of uncompressed collagen gels demonstrated the behaviour of a substrate significantly weaker in stiffness than compressed gels as shown by large differences in elastic moduli. These findings, along with those confirmed during SEM analysis, provide evidence that the compressive properties of collagen gels can enhance substrate stiffness and improve substrate surface topography **(Fig. 1b)** creating a surface similar to that of the bovine cornea **(Fig. 1a)**.

The behaviour of viscoelastic materials is dependent upon conditions such as temperature, pH and hydration (Mezger, 2006) therefore these parameters were kept constant throughout rheology measurements. Changes in the mechanical properties of our materials can be attributed to changes within the structural network of collagen matrices. Having confirmed differences in the stiffness between compressed and uncompressed collagen gels we sought to establish a link between collagen substrate stiffness and stem cell differentiation. Following 21 days culture, LEC expanded on the surface of collagen gels were probed for markers against LEC differentiation (CK3) and un-differentiation (CK14) by immuno-florescent microscopy and Western blotting.

Immunohistochemical analysis **(Fig. 3a.)** of our laminin coated tissue constructs showed intense CK3 expression in the superficial layers of LEC expanded upon stiff compressed collagen gels following 3 weeks in culture whereas CK3 expression was less evident on the less stiff uncompressed collagen gels. Conversely, a cytoskeletal marker of un-differentiated cells CK14 was shown to be expressed in LEC cells expanded upon the uncompressed collagen substrates whilst cells expanded on compressed substrates showed less CK14 expression. It was also observed that tight junction marker ZO1 was evident throughout compressed collagen tissue constructs indicating that stiffer gels facilitate greater cell attachment. Quantification and confirmation of the observed divergent levels of LEC differentiation on compressed and uncompressed collagen gels was ascertained by Western blotting **(Fig. 3b)** where relative band density for CK3 expression on compressed collagen gels is significantly higher (94%) than that on uncompressed collagen gels (6%) . Although previous work described by Mi et al has shown CK3 expression in LEC on uncompressed as well as compressed collagen gels these observations were noted following airlifting, a process which forces cells to and stratify and to differentiate (Chen et al., 2010). In the present study limbal stem were not exposed to airlifting thus ensuring that cells were not forced to differentiate independently of substrate stiffness.

Our immunostaining results can be compared with previous studies showing similar markers in the bovine cornea. CK3 is typically expressed throughout the central cornea with no expression in the limbal basal cells and CK14 is significantly expressed in the basal region of the limbus but is not present throughout the central cornea (Mi et al., 2010b; Zhao et al., 2008). **Figure 3a** shows intense CK3

expression on the superficial layers of our compressed tissue constructs similar to expression in the bovine limbus (Mi et al., 2010b; Zhao et al., 2008). However uncompressed collagen tissue constructs showed CK14 expression throughout, although intense staining is shown in both the superficial and basal layers **(Fig. 3)**.

In addition to stem cell differentiation we investigated the effects of collagen gel stiffness on limbal epithelial cell stratification and number **(Fig. 3c & d)**. Both collagen substrates facilitated dense cell layers following 21 days in culture, however there was a significant increase in cell number on the stiffer compressed collagen gel compared with the less stiff uncompressed gel ($p<0.001$ week two and week three). LEC expanded on the uncompressed collagen substrate in fact showed a decrease in cell number at week two indicating that following an initial seeding density of 0.7 x 10^6 cells were poorly attached on the less stiff collagen gel as shown by weak ZO1 expression **(Fig. 3a)**. It was also observed that the percentage cell viability was higher at weeks two and three on stiff than less stiff collagen gels.

It has previously been shown that limbal epithelial cells are sparsely distributed on uncompressed collagen gels (Mi et al., 2010b) and our results supports this **(Fig. 3a)** showing weak ZO1 expression by LEC on uncompressed collagen gels. ZO1 is known to play an important role in the maintenance of epithelial cell growth and differentiation (Balda et al., 2003; Ban et al., 2003; Ryeom et al., 2000) and is expressed throughout the superficial cells of the corneal epithelium (Ban et al., 2003). Our findings demonstrate increased ZO1 expression by LEC on compressed collagen gels throughout basal and superficial cell layers showing a tightly packed and well stratified corneal epithelial sheet. Although LEC on uncompressed gels are

stratified **(Fig. 3a)** our results indicate cell stratification to be independent of cell density.

It is evident that within our model, compressed collagen gels play a key role in driving limbal stem cell growth and phenotype. Our results suggest that these changes are due to the increased mechanical properties of compressed collagen gels with improved surface nanostructure. More importantly LEC appear to have an increased capacity for growth on stiff compressed collagen gels compared with less stiff uncompressed collagen gels without airlifting. This compares with similar findings in the literature (Hadjipanayi et al., 2009; Haugh et al., 2010; C. R. Lee et al., 2001) and although our substrates facilitate the expansion of four or more cell layers, analysis of cell number at weeks two and three suggests that stiff substrates are better at facilitating cell growth, supported by ZO1 expression related to the presence of tight junctions.

Conclusion

Within tissue engineering there is an increasing demand for substrates with well-defined mechanical and surface properties (Wong et al., 2004). Collagen gels offer several marked advantages over alternative synthetic materials as it is of one of the most abundant proteins in the extracellular matrix (nature's own substrate), is highly biocompatible, biodegradable and exhibits low levels of immunogenicity (Bell et al., 1979; Weiss, 1988). In this study we have shown that collagen substrates with improved mechanical properties and surface structure give rise to tissue constructs with increased levels of cell differentiation and cell number. Our findings demonstrate the importance of factors such as cell differentiation in generating ocular surface constructs. This will have a substantial influence on the future of corneal surface restoration. More importantly our study introduces the possibility of designing tissues capable of imposing precise levels of cell differentiation which will have substantial benefits for the future of regenerative medicine, biomaterials and tissue engineering.

Figure Legends

Figure 1 Characterization of uncompressed and compressed collagen gels. Scanning electron micrographs of **(a)** denuded bovine corneal stroma **(b)** compressed collagen gel **(c)** uncompressed collagen gel. Arrows indicate differences in contrast due to an irregular surface.

Figure 2 Rheology data showing the viscoelastic properties of collagen gels **(a)** Preliminary oscillatory stress sweeps carried out at a fixed frequency of 2π radians between 0.1-100 Pa at 37 °C and **(b)** Oscillatory frequency sweeps carried out under a controlled stress of 10 Pa for compressed gels and 1 Pa for uncompressed collagen gels. Closed symbols refer to storage modulus, open symbols refer to loss modulus.

Figure 3 Protein expression and stem cell analysis on collagen gels. **(a)** Representative immunohistochemical staining of bovine limbal epithelial cells (stained with DAPI, blue or PI, red) on compressed and uncompressed collagen gels. Markers for corneal epithelial cell differentiation (CK3), un-differentiation (CK14) and tight junction (ZO1) are stained green. Arrows indicate the cell-collagen interface and is representative of all sections. **(b)** Western blotting results confirmed and refined CK3 and CK14 immunohistochemical results. CK3 (64 kDa) was strongly expressed in LEC cultured on compressed collagen gels with a higher elastic modulus. In comparison LEC expanded upon the less stiff substrates showed no CK3 expression with a correspondingly high level of CK14 (50 kDa) GAPDH (37 kDa) is shown as a loading control **(c)** The number of LEC on compressed collagen gels is significantly higher than those on less stiff gels ***$p<0.001$ at weeks two and three. Cell viability is

represented as a percentage at each time point. **(d)** Number of cell layers on stiff and less stiff gels following 21 days in culture. P>0.05 and is not significant (-).

Materials and Methods

Preparation of collagen gels

Uncompressed collagen gels were prepared as previously described (Brown et al., 2005) with some modifications. Briefly, collagen gels were prepared by neutralising 4 ml sterile rat-tail type I collagen (2.2 mg/ml in 0.6% acetic acid, First Link Ltd) in 1 ml modified Eagle's minimum essential medium (Fisher Scientific) and 0.5 ml 1 M sodium hydroxide (Fisher Scientific). The solution was gently mixed and cast into rectangular moulds (33 mm x 22 mm x 8 mm) prior to gelling at 37 °C, 5% CO_2 for 30 min. Compressed collagen gels were prepared as above before compressing between a layer of nylon mesh (50 µm mesh size) under 134 g for 5 min at room temperature.

Scanning Electron Microscopy

Collagen gel samples and denuded bovine corneal stroma were examined using scanning electron microscopy (SEM; FEI Quanta FEG 600). Samples were fixed in 2.5 (vol/vol)% glutaraldehyde overnight 4 °C and washed with distilled water. Collagen gel samples were then post-fixed in 1 (vol/vol)% aqueous osmium tetroxide for 2 hours, washed with distilled water then dehydrated in graded ethanol (25%, 50%, 70%, 90% and 100%) prior to critical point drying and gold sputter coating.

Rheology

The rheological properties of compressed and uncompressed collagen gels were determined using a controlled stress AR-2000 rheometer (TA Instruments). Plate-plate geometry (diameter 20 mm) was used for all samples. Preliminary oscillatory stress sweeps were performed to determine the linear viscoelastic region of our

substrates thus ensuring that the moduli were independent of stress. Following this, frequency sweeps were performed with the machine in oscillatory mode over an angular frequency range (ω) of 0.1-100 rad /s.

Isolation of bovine limbal epithelial cells

Bovine eyes (n=10) were obtained from a local abattoir (Chity wholesale abattoir, Guildford UK) within 2 hours of death and transported to the laboratory at 4 °C. Bovine corneoscleral buttons were dissected as previously described (Mi et al., 2010a). Limbal rims (anatomical area around the outer edge of the cornea containing an enriched population of adult stem cells) were then cut into small pieces approximately 25 mm^2 and incubated in 0.02 (wt/vol)% collagenase (Fisher Scientific) at 37 °C overnight. The limbal epithelium was then carefully peeled away from the remaining tissue and treated with 0.25 (wt/vol)% trypsin (Sigma-Aldrich) and 0.02 (wt/vol)% ethylenediaminetetraacetic acid (Sigma-Aldrich) at 37 °C for 1h prior to being aspirated through a 4 mm needle in order to isolate single limbal epithelial cells. Cells were re-suspended in DMEM Ham's F12 medium (DMEM/F12 1:1), supplemented with B27 (Invitrogen), 10 (vol/vol)% fetal bovine serum (Hyclone, Fisher Scientific), 10 ng/ml epithelial growth factor (Sigma-Aldrich), 5 mg/ml insulin (Sigma-Aldrich), 1 IU/ml penicillin and 100 mg/ml streptomycin (Fisher Scientific).

Expansion of limbal epithelial cells on collagen gels of different stiffness

Uncompressed and compressed collagen gels were transferred to polyester membrane transwell inserts held inside six-well culture plates (SLS). The upper surfaces of the collagen gels were coated with laminin (1.5 μg/cm^2, Sigma-Aldrich) for 2 hours prior to cell seeding. 1 ml suspensions of freshly isolated limbal epithelial

cells were seeded onto laminin-coated collagen scaffolds at a density of 0.7 x 10^6 /ml (n=4). Cell culture medium (as defined above) was replenished every 2 days. After 3 weeks incubation at 37 °C under 5% CO_2 the resulting stratified corneal epithelial sheets (tissue constructs) were ready for further examination.

Immunohistochemistry

The tissue constructs were examined by immunofluorescence microscopy to probe for the expression of CK3 a well-established and robust marker of differentiated corneal epithelial cells and CK14 a marker associated with undifferentiated basal corneal epithelial cells. The tissue constructs were embedded in optimal cutting temperature (OCT, TissueTek, Agar Aids) compound and frozen at -80°C. Cryostat sections (7-10 µm thick) were collected onto polylysine coated slides and allowed to air-dry for 2 hours. Slides were then fixed at -20 °C in 100% methanol for 15 min and 100% acetone for 5min prior to incubation with 1 (wt/vol)% bovine serum albumin (BSA) (First Link Ltd) at room temperature to block non-specific binding sites. Sections were then incubated overnight at 4 °C with primary antibodies against CK14 (1:50 guinea pig anti-CK14, Progen), CK3 (1:100 mouse anti-CK3, Millipore) and ZO1 (1:50 mouse anti-ZO1, Invitrogen). Following, sections were incubated with Fluorescein isothiocyanate (FITC)-labelled secondary antibodies (1:50 anti-mouse and 1:100 anti-guinea pig, Serotech) for 1 hour at room temperature. Tissue sections were counterstained with propidium iodide (PI) (Sigma-Aldrich) or 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and analysed by fluorescence microscopy (Carl Zeiss Meditec).

Western Blotting

Proteins extracted from LEC grown on compressed and uncompressed collagen gels (2.5 µg total protein for each condition; estimated using a modified Lowry assay) were separated by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis using 10% gels. Proteins were transferred to polyvinylidine difluoride membranes and nonspecific binding to membranes was blocked by incubation with 5 (wt/vol)% BSA dissolved in 1x TBS-Tween (TBS-T) (20mM Tris-base, 0.14M NaCl, 0.1% Tween -20; pH 7.6). Membranes were incubated with antibodies specific to CK3, CK14 and GAPDH (Abcam) as a loading control (1 µg/ml), diluted in 2 (wt/vol)% BSA dissolved in 1x TBS-T at 4 °C overnight. Blots were washed for 45 min in 1x TBS-T before incubation with anti-mouse or anti guinea pig conjugated secondary antibody (1:4000 dilution or 1:6000 respectively) for 2 hours at room temperature. Proteins were detected on X-ray film using an enhanced chemiluminescence system (Thermo Scientific). Band densities were quantified using ImageJ analysis software.

Examination of cell number and stratification

LEC stratification was determined by the double-blind counting of cell layers (n=6) from light microscope images of epithelial cells on compressed and uncompressed collagen gels taken at 20x magnifications. To determine cell number LEC were seeded at a density of 0.7 x 10^6 /ml on each scaffold. At weeks two and three cells were harvested and counted using Trypan blue (Hyclone, Thermo Scientific). A two-tailed t-test (unpaired) was performed to determine the statistical significance of the number of LEC layers and total cell number on compressed and uncompressed

collagen gels using Microsoft Excel. Results are presented with a standard deviation of the mean where $p \leq 0.05$ was considered significant.

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Competing interests statement

The authors declare no competing financial interests

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